**Full-length PAR-2 purification**

We grew 4L of High Five insect cells, and infected cells with virus packaged with pNH0046, a vector containing FLAG-StrepII2X-tagged PAR-2. This vector was built by Nisha Hirani, by inserting the full-length PAR-2 sequence into pASC144 (based on pFBDM), a vector designed for expression of recombinant proteins in insect cells. The virus was prepared by Colin Davis.

Infected cells were incubated at xC for 3 days, centrifuged, and pellets frozen at -80C. We then resuspended pellets in lysis buffer (recipe in table x) and sonicated samples on ice. We then centrifuged sonicated samples and discarded pellets. Next, we performed strep pull-down on the supernatant using a StrepTactin column, and eluted strep-tagged protein with elution buffer (recipe in table x). We followed this with size exclusion chromatography using a Superdex 200 column.

**Ubiquitination assays**

**PAR-2 RING domain fragment purification**

Based on secondary structure prediction and sequence alignment, we identified amino acids 40-120 of PAR-2 as sufficient to contain the core RING domain and flanking dimerisation helices. I amplified this sequence from pNH46 using primers x and x and cloned into the pETM11 His-Sumo vector (obtained from the Crick Structural Biology STP), generating the plasmid pTB10, which I transformed into Rosetta (DE3) cells by electroporation.

I grew 5ml overnight cultures of transformed cells and used these to inoculate 12x 500ml LB supplemented with 50mM zinc sulphate. Cells were grown in a shaking incubator at 37C to an OD600 of 0.6. At this point, we reduced the temperature to 16C, added 100uM IPTG and left cultures shaking overnight. The following day, we centrifuged the cultures and froze pellets at -80C until ready for subsequent steps.

We began subsequent purification by resuspending pellets in lysis buffer containing protease inhibitors (recipe in table x) and sonicating samples on ice. We then centrifuged sonicated samples at high speed and discarded the pellets. We used the Ni-NTA agarose kit from Qiagen to pull His-tagged protein from the supernatant onto beads, and washed beads with lysis buffer without protease inhibitors, before eluting protein off beads with elution buffer (recipe in table x).

Next, to cleave the His-Sumo tag from the protein, we added the SUMO protease SenP2 (obtained from the Crick Structural Biology STP) at an approximate ratio of x, and left the sample overnight at 4C whilst dialysing in TBS with x dialysis tubing. We then removed cleaved tag from the sample using the Ni-NTA agarose kit.

To remove any impurities from the sample, we performed ion exchange chromatography using x and size exclusion chromatography using x.

To purify mutants of the RING domain, I performed site-directed mutagenesis on pTB10. For L109R I used primers x and x, generating the plasmid pTB13. For C56S I used primers x and x, generating the plasmid pTB11. We attempted to express and purify 1L (check) samples of both mutants. Whilst both were expressed in cells, we found that C56S was unstable during the purification process and we were unable to purify large quantities. L109R, on the other hand, was more stable, and we managed to purify sufficient quantities for one SEC MALS run.

**SEC-MALS**

Samples were run over a size exclusion chromatography using a x column, and analysed by multi-angle laser light scattering (MALLS) using x.